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Ricin is a cytotoxin and a known bioterrorist weapon. The Army is pursuing anti-ricin vaccines, but plans to develop an efficacious antidote to the toxin, for cases where vaccination is not appropriate. The goal of this project is use the X-ray structure of ricin A chain (RTA) as a template for inhibitor design. Computer modeling and X-ray screening aid in the design process. Inhibitors which bind to the RTA substrate specificity site have been identified. A previous platform, 9-oxoguanine, has been shown to be of limited synthetic utility, however, 9-deaguanine appears to be a more versatile specificity site platform and should allow a range of pendant additions. A search has been initiated to screen for pendants which can be attached to the platform and which make strong and specific interactions at other sites. These groups can aid inhibitor uptake, binding specificity, and binding strength. A new compound, based on aminimide synthesis, was tested just as this report was being prepared. It has an IC_{50} more than 1000 times as potent as any past inhibitors we have tested; this finding will cause us to modify our research direction to some extent.

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Table of Contents

INTRODUCTION

This contracted project is aimed at designing, synthesizing, and testing small molecule inhibitors of the cytotoxin ricin. Ricin is a class B biological agent which is known to be in the possession of terrorist groups (Loyd and Fletcher, 2001). Although not as menacing as infectious agents, ricin is of great concern because of the ease with which large amounts of semi pure material can be produced (Wellner, et al, 1995). The Army is proceeding with vaccine development for key military personnel (Olson et al, 2004). However, wide spread vaccination of the military or civilian population is not practical or desirable, and so there is a need for an efficacious antidote. In addition to its utility such a compound could reduce panic that arises from a relatively minor terrorist incident. Our area of expertise is the rational design of inhibitors of enzymes like ricin A chain (RTA). We have elucidated the three-dimensional structure of ricin and this model serves as a template for the design of small molecules that can bind tightly and inactivate the toxin. These inhibitor compounds should also incorporate elements of drug design, including solubility, stability, and low toxicity. We have used computer searches to identify classes of inhibitors that act as "platform" molecules. These platforms have been modified and appended creating novel inhibitors for RTA. This particular project is a collaboration between structural biologists (Robertus group) and synthetic chemists (Kerwin group) to extend our previous research efforts on antidotes. It is a step-wise process, beginning with modest inhibitors, which are then sequentially improved after analysis, to produce ever more potent compounds; the program is scheduled to last three years. Our overall goal is to create a ricin inhibitor which is efficacious at inhibiting ricin intoxication of cultured cells, and itself is non-toxic. Since an aerosol dispersion is considered the most likely form of attack we (Kehrer group) will use cultured human lung cells for this test.

BODY

The original Statement of Work (SOW) is as follows:

Task 1: Design improved specificity pocket (months 1 - 12)
- a. Prepare 9-oxaguaninine
- b. Prepare other related heterocycles

Task 2: Identify ligands for second ricin binding pocket (months 1 - 18)
- a. Use computer searches based on ricin structure
- b. Apply crystallographic screening of shape-diverse sets of commercial compounds

Task 3: Prepare tripartite inhibitors joining best specificity pocket and second pocket moieties with appropriate linkers (months 9-24).

Task 4: Use iterative crystallographic algorithm to refine tripartite inhibitors (months 12-36)
- a. Modify compound shape to fit enzyme contours
- b. Design compounds for maximum water solubility and biological uptake

Task 5: Test biological efficacy of inhibitors as ricin antidotes (months I-36)
- a. Candidate inhibitors will be tested against ricin enzyme activity
- b. Ricin inhibitors will be tested for protective action in cultured human lung cells
- c. The most promising ricin antidotes will be sent to a commercial testing facility for initial human safety tests using a panel of enzyme and receptor assays.

ACCOMPLISHMENTS FOR YEAR 1

Year one efforts are primarily concerned with Tasks 1 and 2 in the SOW, although a start has been made on Task 3. In addition, we needed to modify the RTA enzyme assay, which can be called Task 0.

TASK 0: Modifying and recalibrating the ricin enzyme assay.

In the past our laboratory has measured ricin activity by its ability to inhibit the synthesis of radiolabeled poly Phe on ribosomes from *A. salina*, using transcription factors purified from wheat germ (Ready et al, 1983). When we restarted work on RTA inhibitors we found this assay was no longer feasible. We investigated commercial protein synthesis kits centered around rabbit reticulocyte ribosomes, testing several messages. However because of cost and ease of use we adopted the simplest assay in which reticulocyte ribosomes express natural hemoglobin message. Because the ribosomes are preloaded with message, and may form RTA resistant polysomes, even the most aggressive attacks by RTA cannot eliminate all background synthesis. However, the background rate is controllable and a simple dose response for a 30' incubation of RTA with the reticulocyte system at 30 °C is shown in Figure 1. The IC_{50} for RTA is 0.15 nM, which compares favorably with the value of 0.3 nM seen previously with the A. salina system (Ready et al, 1991).

To further confirm the utility of the new system, we used it to measure the inhibition of RTA by pteroic acid (PTA), a known inhibitor of the enzyme. For this test we fixed the concentration of RTA at 0.15 nM. The results are shown in Figure 2. The IC_{50} of PTA for RTA is about 0.55 mM, which, again, compares favorably with the value of 0.6 mM seen with the A. salina system (Yan et al, 1997).

Finally, we used the new assay to measure the inhibition of RTA by 8-methyl-9-oxoguanine (9OG). This novel compound of our design had been the most potent small molecule "platform" inhibitor previously tested (Miller et al, 2002). 9OG was resynthesized just prior to testing and the results are shown in Figure 3. The IC_{50} is roughly 40 μM, about 10 times better than the 400 μM reported earlier (Miller et al, 2002).

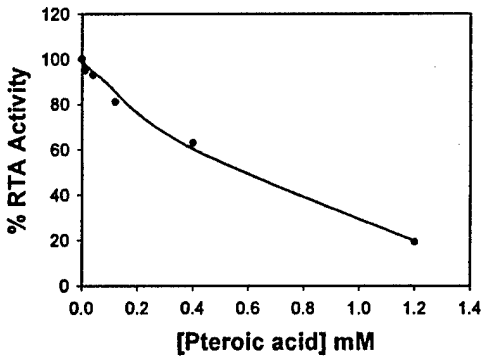


Figure 3: Inhibition of RTA by pteroic acid in the reticulocyte assay.

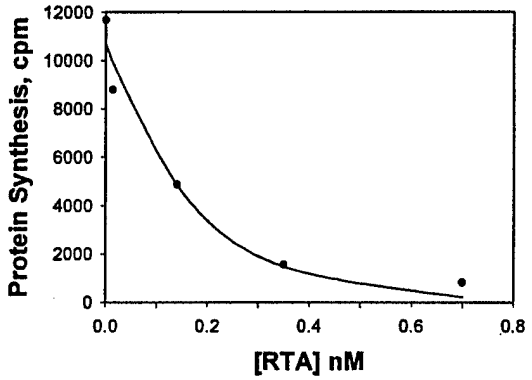


Figure 1: A dose response curve for RTA against a commercial reticulocyte lysate kit.

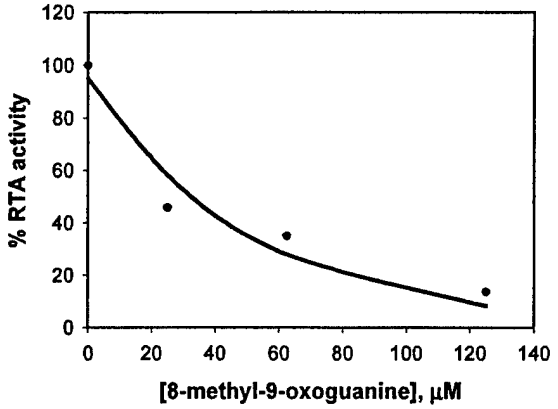


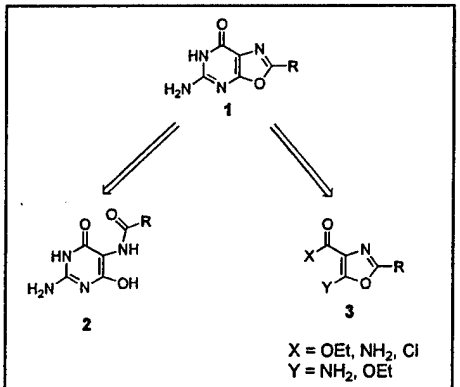
Figure 2: Inhibition of RTA with 9OG using the reticulocyte assay.

In addition to establishing the reticulocyte based protein synthesis assay, we also tested a simple aniline cleavage assay for RTA activity. This assay has been used by others for qualitative measures of N-glycosidase action (Endo et al, 1987). We observed that some potential RTA inhibitors can also inhibit ribosomes, thereby rendering the protein synthesis assay useless (Miller et al, 2002). The aniline cleavage assay only depends on the 28S rRNA being intact; depurination by ricin renders the chain susceptible to aniline cleavage which can be observed as a novel band on an acrylamide gel. We used yeast ribosomes as a source of rRNA and showed a dose response activity of RTA against it. Our experiments (not shown) suggest that the aniline assay can only give qualitative results. That is, it might be able to rank order inhibitor strengths, but it is too noisy to give reliable IC_{50} measures.

TASK 1: Design improved specificity pocket ligands (months 1-12)

A substantial portion of the synthetic work carried out over the last 12 months has focused on the construction of heterocyclic scaffolds that have been predicted to bind to the specificity pocket of the catalytic ricin A-chain. These efforts have resulted in the preparation of examples of an entirely new class of heterocycles (7-deaza-9-oxaguanines), a previously reported versatile intermediate for the preparation of 7-substituted-9-deazaguanines, and numerous precursors to substituted 9-oxaguanine derivatives. However, we have been unable to prepare any 9-oxaguanine other than our previously reported 8-methyl-substituted analogue. At this point, we have not been able to improve upon this specificity pocket ligand.

A. Prepare 9-oxaguanine analogues



Scheme 1. Retrosynthetic analysis of the 9-oxa-7-substitutedguanine analogs.

Our previous work had demonstrated that 8-methyl-9-oxa-guanine (**1**, R = Me, Scheme 1) inhibits ricin by binding to the specificity pocket. While the inhibition constant for 8-methyl-9-oxa-guanine was rather weak, we believe that analogs that contained more suitable side chains will be more effective inhibitors. Two potential approaches to the 9-oxa-guanine ring system were proposed (Scheme 1). In one approach, an acylated diaminohydroxypyrimidine (**2**) undergoes cyclodehydration to form the oxazole ring of the 9-oxa-guanine heterocycle. In another approach, the oxazole ring is constructed first (**3**), and the pyrimidine ring is subsequently elaborated. We have examined each of these routes, as described below. The preparation of the methyl substituted 9-oxa-guanine derivative (**1**, R = Me) was originally carried out through cyclodehydration of the 5-acetamido-2-amino-4-hydroxypyrimidine (**2**, R = Me). A variety of cyclodehydration conditions were tried, but heating the solid acylaminopyrimidine (**2**, R = Me) under vacuum and collecting the product by sublimation was the only method that was found to reproducibly afford 8-methyl-9-oxaguanine (**1**, R = Me) in useful yields. Thus, our original efforts focused on this

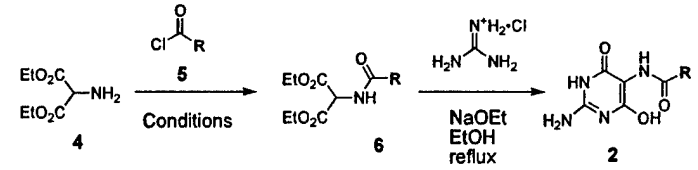
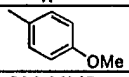
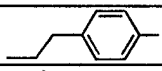
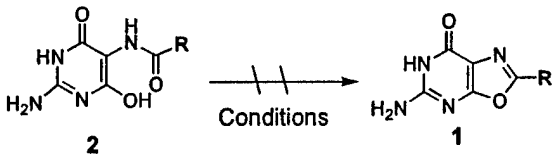


Table 1. Preparation of 5-acylamino-2-amino-4-hydroxypyrimidines

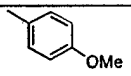
	5, R =	Conditions	Yield 6	Yield 2
a	CH_2CH_2Ph	K_2CO_3, CH_2Cl_2	98%	22%
b	$CH(CH_3)_2$	$NaHCO_3$, ether/H_2O	92.5%	82%
c		Et_3N, THF	85%	71%
d		Et_3N, THF	79%	58%
e	CH_2NHBoc	DCC, HOBT, THF*	82%	64%
f		DCC, CH_2Cl_2*	63%	n/a

* from the corresponding carboxylic acid.

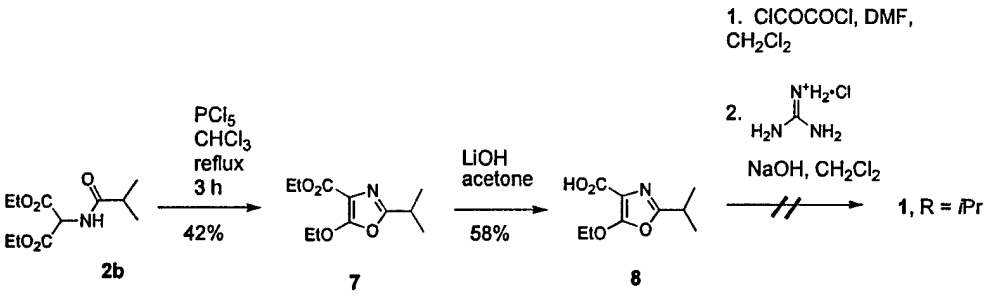


2 1

Table 2. Attempted cyclodehydration of 5-acylamino-3-amino-4-hydroxypyrimidines.

	2, R =	Conditions
a	CH_2CH_2Ph	300 °C, 0.8 mm Hg, 24 h or 280–300 °C, 1 atm Ar, 14 h
b	$CH(CH_3)_2$	280–300 °C, 0.8 mm Hg, 18 h or POCl3, reflux, 8 h or PPA, 120–150 °C, 8–14 h
c		300 °C, 0.8 mm Hg, 20 h or PPA, 70–180 °C, 8 h
d		280–300 °C, 0.8 mm Hg, 18 h

procedure, starting with a variety of 5-acylamino-2-amino-4-hydroxypyrimidines **2** (Table 1). Despite our previous successful cyclodehydration of 5-acetamindo-2-amino-4-hydroxypyrimidine (**2**, R = Me) to 8-methyl-9-oxa-guanine (**1**, R = Me), we have been unable to prepare any other 9-oxaguanine analogs through this route (Table 2). One possible reason that the sublimation conditions that were successful in affording 8-methyl-9-oxaguanine fail in the other cases due to the decreased volatility of the 9-oxa-guanines. That is, if any of these 9-oxa-guanine derivatives form, they decompose at the temperatures required for their sublimation. Thus, we turned to alternate procedures, including heating solid 2 under



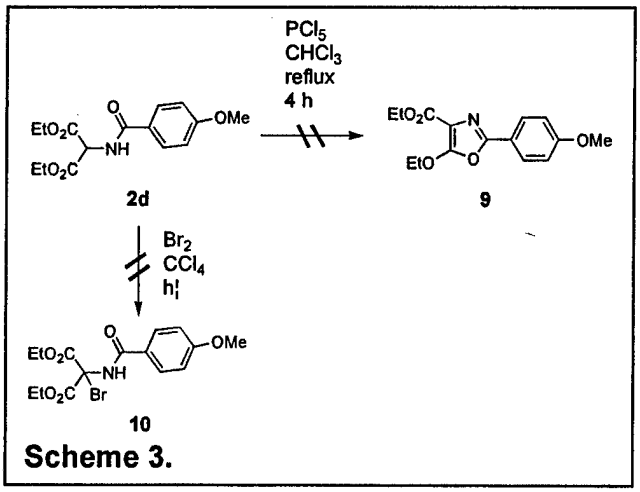
2b 7 8

Scheme 2.

an atmosphere of argon or under vacuum, or cyclodehydration of **2** with polyphosphoric acid (PPA) or phosphorous oxychloride; however, none of these methods were successful (Table 2).

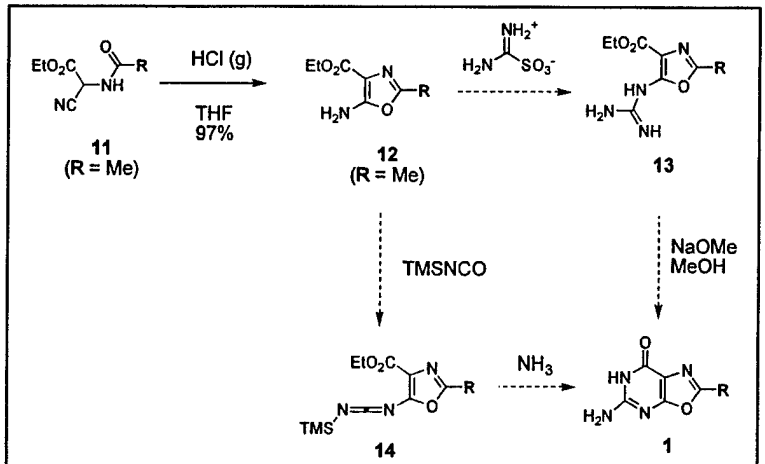
We next turned to the alternative route to these 9-oxaguanine derivatives, which involves elaborating the pyrimidine ring after formation of the oxazole ring. This route has some precedence in the literature, (Patil et al, 1971; Turchi et al, 1983; Sekeya et al, 1990) but no cases have been reported in which 2-amino-substituted heterocycles (such as **1**) were formed. Our efforts have currently focused on intermediate 4-carboxy-5-ethoxyoxazoles. Thus, the acylated aminomalonate **2b** undergoes cyclodehydration with PCl_5 in refluxing chloroform to afford the oxazole **7**, which is subjected to careful hydrolysis with LiOH to give the carboxylic acid **8** (Scheme 2). Our initial efforts to couple this carboxylic acid with guanidine, followed by cyclization have not been

successful. We will continue this approach by exploring other coupling methods, including direct coupling of ethyl ester **7** with guanidine in sodium methoxide/methanol. As shown in Scheme 3, we have also met some difficult in formation of other oxazoles. Despite the successful conversion of **2b** to **7** with PCl_5, the aminomalonate derivative **2d** did not afford the oxazole **9** under similar conditions. We have begun to explore alternative routes to the oxazole **9**. Attempts to brominate **2d** did not afford the bromomalonate **10**; rather, electrophilic aromatic bromination takes place. Our plan is to employ a free radical initiator such as AIBN in this reaction in the future in order to bias the products towards those derived from free radical bromination. We have also begun to construct precursors to more elaborate oxazoles. The acylaminomalonate **6f** (Table 1) has been prepared and will be subjected to cyclodehydration or bromination/cyclization conditions in order to afford the corresponding oxazole.

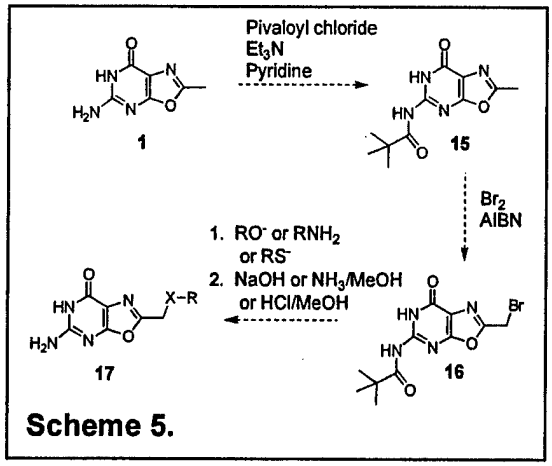


Scheme 3.

If we continue to have difficulty with this route to 9-oxa-guanine derivatives, we will explore an alternative route from 4-amino-oxazole derivatives (**3**, Y = NH_2, Scheme 1). These can be prepared from the corresponding aminocyanomalonates, as shown below for **11**, which we have used to prepare the amino-oxazole **12** (Scheme 4). Such amino-oxazoles will be treated with a formamidinylating reagent to give the substituted guanidines **13**, which will undergo cyclization to 9-oxa-guanines **1**. Alternatively, conversion of these to phosphorimides followed by reaction with trimethylsilylisocyanate may give the carbodiimides **14**, which may cyclize to 9-



Scheme 4. Preparation of 5-aminooxazoles and planned conversion to 9-oxaguanine derivatives.



Scheme 5.

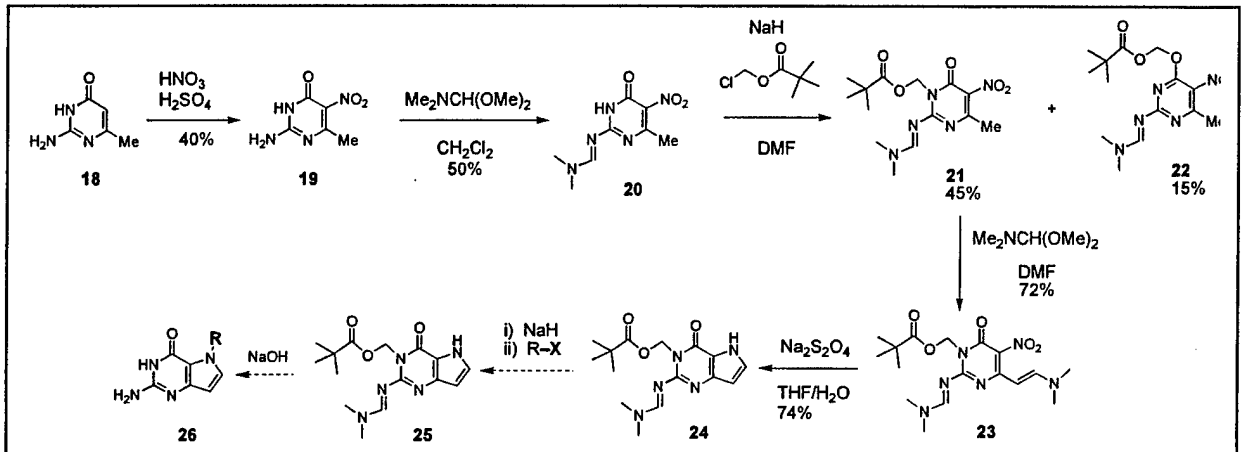
oxa-guanines upon treatment with ammonia (Scheme 4).

Finally, we are exploring an alternative route to 8-substituted 9-oxa-guanine derivatives through the 8-methyl compound **1**. We will protect the exocyclic amino group of **1** as the pivaloyl amide **15** (Scheme 5), followed by free-radical bromination of the methyl group to afford the bromomethyl derivative **16**. This bromomethyl derivative can then be elaborated to afford a variety of 8-substituted-9-oxa-guanine derivatives (**17**) Scheme 5.

B. Prepare other related heterocycles

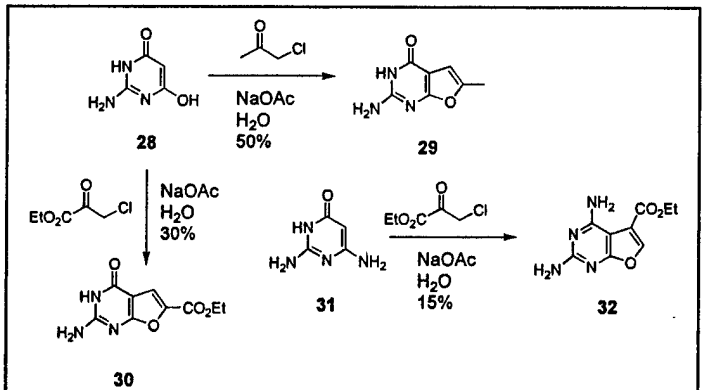
i) 9-Deazaguanine derivatives.

We had also identified 9-deazaguanine as a promising platform inhibitor for ricin; however, we wanted to prepare substituted analogs in order to probe the effect of substituents on the orientation of the compound in the specificity site as well as the



Scheme 6.

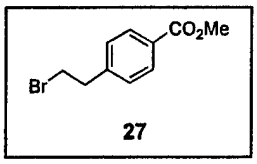
affinity for ricin. We have prepared the key intermediate **24** (Scheme 6), following the route of Taylor and Young (Taylor and Young, 1995). Alkylation of **24** with a variety of alkylhalides (R-X) followed by NaOH deprotection should afford the 7-substituted 9-deazaguanine deriviatives **26**. Our initial attempts using the alkylbromide **27** have been met with very low yields of alkylated product, due to the facile elimination of HBr from this compound under the alkylation conditions. We are optimizing the reaction conditions employing **27**, as well as exploring a variety of alternative alkylhalides in this reaction.



Scheme 7.

ii). 9-Oxa-7-deaza-guanines

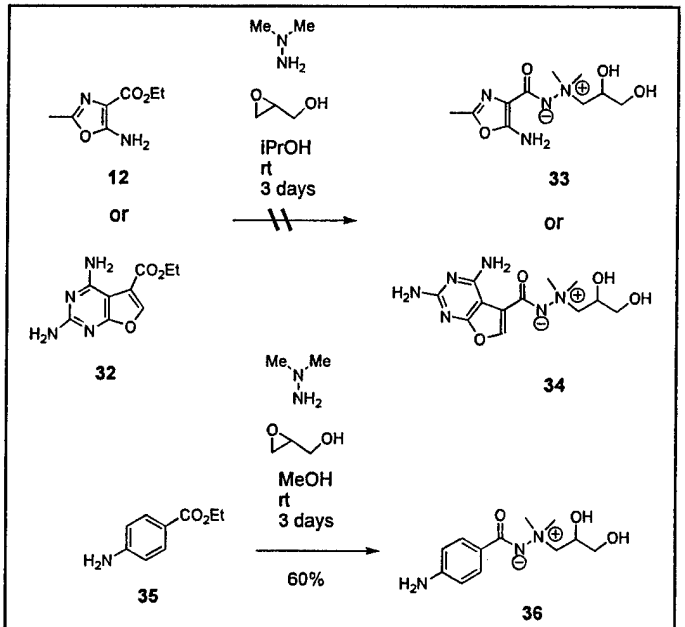
A series of 7-deaza-9-oxaguanine analogs (2-amino-3*H*-furo[2,3-*d*]pyrimidin-4-ones) have been prepared in order to explore this heterocyclic framework as an alternative platform for ricin inhibition. Condensation of chloroacetaldehyde or ethyl chloroformate with 2-amino-4,6-dihydroxypyrimidine (**28**) affords the corresponding 8-substituted 7-deaza-9-oxaguanines **29** and **30** (Scheme 7). In a related reaction, condensation of ethyl chloroformate with 2,4-diamino-6-hydroxypyrimidine (**31**) affords the 9-substituted 7-deaza-9-oxaguanine **32** (Scheme 7).



27

C. Prepare Aminimides

Hydroxyethyl aminimides can be prepared via a three component condensation reaction involving an ester, a 1,1-disubstiuted hydrazine, and an epoxide (Slagel, 1968). The resulting aminimides stable zwitterionic compounds that are water soluble. Due to their structural similarity to peptides, aminimides have been employed as peptide isosteres in the design of inhibitors of proteases (Rutenber et al, 1996; Peisach, 1995). Our interest in these compounds derived from their favorable solubility; we reasoned that the construction of platform heterocycles targeting the ricin specificity pocket with aminimide side chains would lead to both increase affinity for ricin as well as improved water solubility of these inhibitors. We carried out the three component condensation reactions with 1,1-dimethylhydrazine, glycidol, and the platform heterocyclic esters **12** and **32** in isopropanol (Scheme 8). Unfortunately, we did not observe formation of any of the desired aminimide products, possibly due to the presence of an adjacent amino group in both esters. In contrast, condensation reactions carried out with ethyl aminobenzoate **35** afforded the desired aminimide **36** in good yield as a hydroscopic white solid.



Scheme 8.

Inhibition of RTA by the aminimide

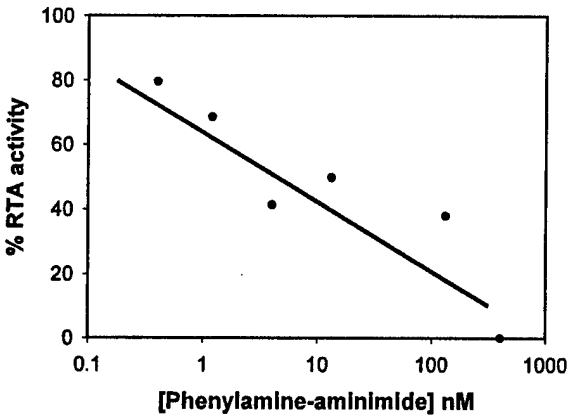


Figure 4: Dose response for the aminimide

Compound 36, referred to as phenylamine-aminimide, proved to be very soluble in water and showed no inhibition of protein synthesis even at 1 mM concentrations. The ability to inhibit RTA activity was very strong, as shown in Figure 4. At this time the data suggest it has an IC_{50} of about 10 nM, making it at least 1000 times as potent as any small molecule inhibitor of ricin seen to date. One concern about the aminimides is that their low lipophilicity might retard cell uptake. At least one study (Boutis et al, 1978) shows aminimides are active against tumor lines, suggesting they can be taken into cells. Further design can be carried out to optimize uptake of this potent class of compounds.

TASK 2: Identify ligands for second ricin binding pocket

A. Computer Modeling studies

We acquired a program called LigBuilder to assist in inhibitor design (Wang et al, 2000). We had previously used the program ChemX which carries out virtual screens. That is it fits pre-existing molecules from a data base to the RTA active site test for stereo chemical fits; we used this program to find pteroic acid for example. LigBuilder has a different philosophy - it is a "de novo" designer. It maps the active site for chemical properties like charge, hydrogen bond patterns, and hydrophobicity. It then fills the active site with simple complementary chemical groups, pharmacophores, from an internal library. It uses a genetic algorithm to search a large space and suggests a list of novel compounds that might bind strongly. It uses several established pharmacological criterion to rank these candidates.

We carried out a number of de novo building runs. One observation was that the program tends to design inhibitors that are too elaborate and would be synthetically difficult. However, the program is still very useful for defining recurrent pharmacophor "themes". For example, hundreds of potential ligands generated by LigBuilder placed an aromatic ring above the side chain of Trp 211, although in slightly different positions and orientations. To test if there is a natural site in the RTA active site for such a ring, we used the energy mimization routine from the X-ray suite CNS/XPLOR (Brunger, 1992). A test ring was positioned at several places identified by LigBuilder, and allowed to refine. The results are shown graphically in Figure 5. The RTA active site area is represented by the side chains of Tyr 80, Trp 211, and Val 256. Five representative benzene ring starting positions are shown as light gray hexagons and their energy refined positions as black hexagons. The tests suggest that the volume can accommodate an aromatic ring in a wide variety of positions and orientations and that there is no well defined global minimum to act as

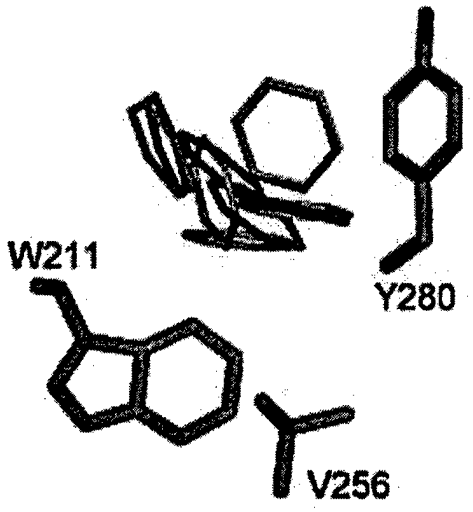


Figure 5: Test of the energetics of a hydrophobic pharmacophore.

a constraint on inhibitor synthesis. An inhibitor will benefit from a hydrophobic group near here, but if need not be in a restricted orientation.

Another interesting pharmacophore is a positively charged group that sits near Glu 177 and Glu 180. We had suggested that the RTA mechanism might involve stabilization of an oxocarbenium ion (Ready et al, 1991), and this contention has been strengthened by the work of Schramm and his colleagues. They synthesized an RNA analog with a positively charged ring group to mimic the transition state and found it bound RTA with high affinity (Tanaka et al, 2001). Consistent with this notion, LigBuilder frequently designed ligands with a positively charged amine group in this area. We designed a simple ligand that incorporated these features: 9-oxo-7 deaza-guanine is the specificity group, a protonatable secondary amino group near the Glu 177 side chain, and a benzene ring in the hydrophobic site (shaded). The name of this compound is: 2-Amino-5-(2-benzylamino-ethyl)-3,7-dihydro-pyrrolo[3,2-d]pyrimidin-4-one, but we refer to it as 9DG-D5 (Figure 6). This compound lacks the pendant groups we eventually plan to add, which will bind to a putative "second pocket" on RTA. 9DG-D5 may be considered as a platform, plus linker, module. Energy minimization with CNS shows that all three moieties can be positioned in the RTA active site without tortional stress.

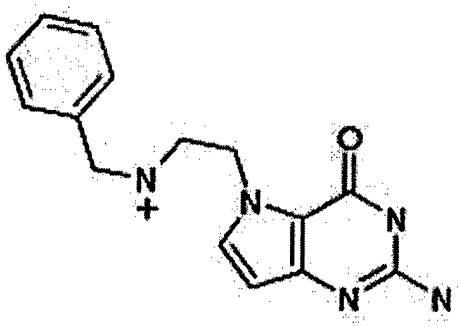
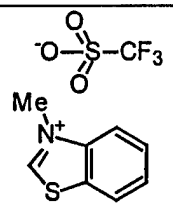
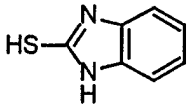
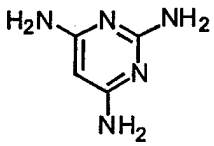
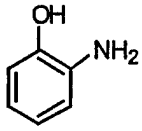
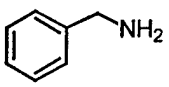
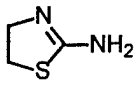
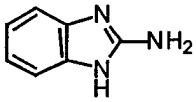
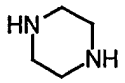
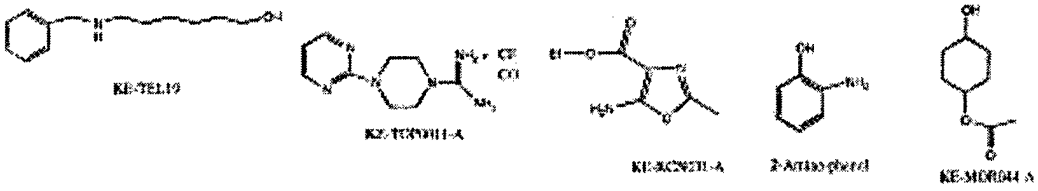


Figure 6: A designed ligand, 9DG-D5

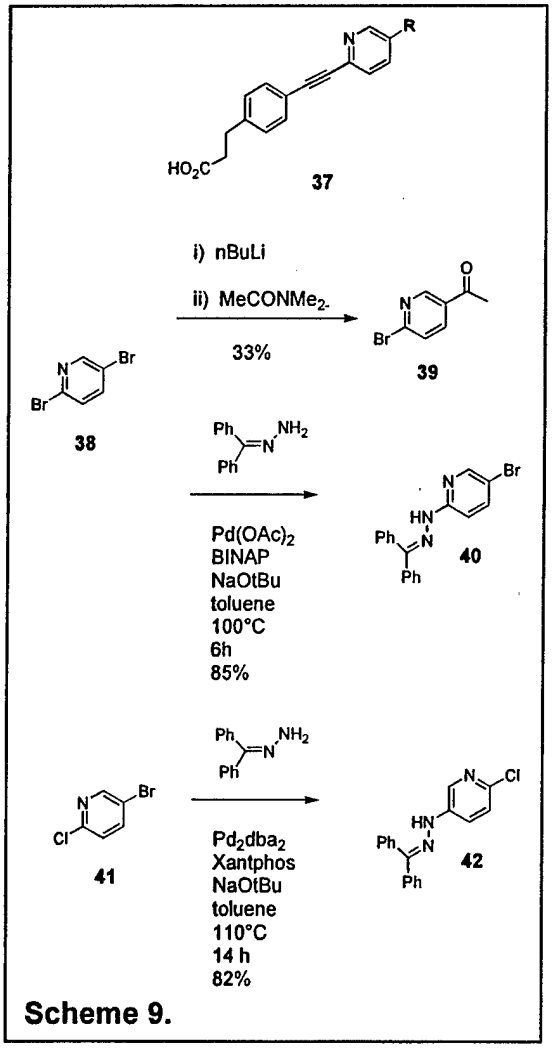
B. Crystallographic screening of shape-diverse sets

We have begun searching commercial compounds using the crystaLEAD, shape-diverse, screening method (Nienaber et al, 2000). We know that RTA has a primary specificity binding pocket which holds the adenine ring of natural substrates, and into which our inhibitor platform molecules bind. We plan to probe the vicinity for additional binding pockets that could interact favorably with pendant groups attached to the platform. We are screening for these pendants by soaking cocktails of potential "second site" molecules into RTA crystals and using X-ray diffraction to see if any bind to the protein. The cocktails are designed so that each has molecules sufficiently different in shape that X-ray crystallography can distinguish which molecule is binding to RTA. To date we have tested three cocktails; typically the stock solution contain 4 or 5 compounds each at 5 mM. These are diluted to roughly mM concentrations in the crystal screen. The three panels are shown below in Table 3. None to the screens has detected a strongly binding compound. The electron density maps show minor difference electron density "traffic" in the solvent areas, but nothing above 3σ inside the protein or in surface crevices.

Table 3: Shape diverse libraries screened by X-ray diffraction				
Shape diverse library #	Shape-diverse Compound Structures			
1	 KeAZB024	 2-mercaptobenzimidazole	 2,4,6-triaminopyrimidine	 2-aminophenol
2	 benzylamine	 2-aminothiazoline	 2-aminobenzimidazole	 Piperazine
3				

TASK 3: Prepare tripartite inhibitors joining best specificity pocket and second pocket moieties with appropriate linkers (months 9-24).

Synthesis of 2,5-Disubstituted pyridines



Scheme 9.

The pyridine-based side chains **37** were identified through computer modeling as potential ligands for the RTA second pocket. We proposed preparation of these side chains through coupling of 2-halopyridine derivatives with a 4-ethynylhydrocinamic acid derivative. We have prepared two such 2-halopyridine derivatives **39** and **42** (Scheme 9). Selective lithium-halogen exchange of 2,5-dibromopyridine (**38**) with butyl lithium, followed by addition of dimethylformamide affords the 2-bromopyridine derivative **40** in modest yield. Palladium-catalyzed coupling of **38** with benzophenone hydrazone leads to selective coupling at the 2-position to afford the undesired product **40**; however, coupling with the bromochloropyridine **41** gives the desired 2-chloropyridine **42** in good yield. Both **39** and **42** will be coupled to 4-ethynylhydrocinamic acid to afford the desired side chains **37** (R= $COCH_3$ and $NHNH_2$)

KEY RESEARCH ACCOMPLISHMENTS

- A modified assay for RTA/inhibitor studies has been established and validated.
- A synthesis for 9-deazazguanine able to accept a range of pendants at C7 has been worked out.
- The synthesis of substituted aminimides has been carried out very recently as a new class of ricin inhibitors.
- Phenylamine-aminimide is the most potent RTA inhibitor yet tested, with an IC_{50} of 10 nM (page 11).
- Computer modeling (LigBuilder) and energy minimization experiments have mapped parts of the RTA inhibitor binding surface
- A successful synthesis of 2,5-Disubstituted pyridines has initiated studies for "second site" inhibitor pendants.

REPORTABLE OUTCOMES

No papers from year one work have yet been prepared, but publishable work has been accomplished. The aminimide inhibitors are particularly exciting.

CONCLUSIONS

Year one initially involved assembling the research teams, reproducing past work with new researchers and then executing the research plan. We discovered that the synthetic strategy which led to our previous best compound, 8-methyl-9-oxoguanine, was not general to pendants other than the 8-methyl group. We compensated by expanding work on other platforms, especially 9-deazazguanine. This new platform was used in modeling experiments to generate a new idealized platform-linker concept; we found the new de novo drug design program, LigBuilder, was simple to use and generate useful insights into chemical classes that interact favorably with RTA. Perhaps the most exciting finding was the potency of the aminimide compounds. These peptide mimetics have been used as peptidase inhibitors. Their modular nature, coupling a wide range of commercially available esters, azides, and epoxides, made them attractive candidates for this project. Their synthesis proved more difficult then expected, however, which may account for the relative paucity of literature data for this apparently flexible chemical class. However, the great potency of our first simple model suggests we should divert some effort to optimizing this class of compounds; the unoptimized phenylamine-aminimide is roughly 4000 times more potent than any other inhibitor of RTA we have tested. X-ray studies are underway to analyze the binding of phenylamine-aminimide to RTA and to explain its potency. Our current hypothesis is that the aminimide zwitterionic nitrogens, of the form [--N^- -- N+--], may interact with Arg

180 and Glu 177, respectively, in the RTA active site. We presume the X-ray analysis will show the phenylamine group binds in the specificity cavity or RTA; if this is so, we plan to incorportate starting material esters which are known to make favorable interactions with the RTA specificity site.

"**So what**?": The proposed inhibitor design project is making headway at the anticipated rate; that is, efforts to assemble a tripartite inhibitor are on schedule with some successes and some setbacks. The newly discovered, and as yet synthetically unrefined, phenylamine-aminimide has an IC_{50} that is in the range for true drugs. We will undertake studies to test if the compounds are taken up effectively by cells. It may be that future design for this chemical class will change from emphasizing potency against RTA to improving cell uptake. It seems quite likely that RTA antidotes can be created.

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